Embryogenic suspensions of adult cork oak: the first step towards mass propagation

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Abstract Protocols have been established to clone adult cork oak trees by somatic embryogenesis using semisolid medium. However, for economically viable mass propagation, embryogenic cultures in liquid medium need to be developed. In this study, suspension cultures were initiated from embryo clusters obtained by secondary embryogenesis on a gelled medium lacking plant growth regulators. After 6 days of culture, these embryo clusters generated high cell density suspensions that also contained small organized structures (embryos and embryogenic clumps). As the culture duration increased, tissue necrosis and fewer embryogenic structures were observed and the establishment of suspension cultures failed. An alternative method was found adequate for initiation of embryogenic suspensions: embryo clusters from gelled medium were briefly shaken in liquid medium and detached cells and embryogenic masses of 41–800 μm were used as inoculum. Maintenance of embryogenic suspensions was achieved using a low-density inoculum (43 mg l^{-1}) by subculturing four embryogenic clumps of 0.8–1.2 mm per 70 ml of medium. Proliferation ability was maintained for almost 1 year through ten consecutive subcultures. The initiation and maintenance protocols first developed for a single genotype were effective when tested on 11 cork oak genotypes.

Keywords Micropropagation · *Quercus suber* · Somatic embryogenesis · Suspension culture

Introduction

Somatic embryogenesis (SE) has potential applications in forest tree genetic improvement programmes because it allows the selection of individuals to be clonally propagated (Merkle and Nairn 2005). Embryogenic systems based on semisolid culture media are generally not useful for the mass production of plant material. However, the efficiency of SE could be improved by culturing cell clumps with embryogenic capacity in a liquid medium. It has been shown that such suspensions give rise to high propagation rates and simplify management practices (Ziv 2000). In addition, embryogenic clumps (EMCs) are ideal candidates for genetic transformation, because the efficiency of transformation and regeneration of transformants are increased (Andrade et al. 2009).

In a model species such as carrot, embryogenic suspensions are usually initiated from callus induced by exposing young tissues, mainly zygotic embryos, to high auxin concentrations. Once the culture was established, the presence of auxins in the medium inhibited embryo development and instead the suspensions underwent recurrent production cycles of small cell clumps that are known as proembryogenic masses, PEMs (Halperin 1966). These PEMs were triggered to develop into somatic embryos by transfer to a medium that either

lacked auxins or contained a reduced concentration of auxins. This commonly used protocol (Finer 1994; Merkle et al. 1995; Osuga et al. 1999; Komamine et al. 2005) was successful in some forest species such as ash (Tonon et al. 2001) and American chestnut (Andrade and Merkle 2005).

In cork oak (*Quercus suber* L.), somatic seedlings were obtained from adult trees through induction of SE in leaves (Hernández et al. 2001; Pinto et al. 2002). The protocol developed by Hernández et al. (2001) was used to induce SE and regenerate clonal plants practically in every cork oak tree in which this was attempted (Hernández et al. 2003b). Presently, field trials and clonal tests with somatic seedlings from select genotypes are being assessed (Celestino et al. 2009; Hernández et al. 2011).

SE was induced in leaves of adult cork oak without the formation of callus and no callus was produced during subsequent proliferation (Hernández et al. 2003a). The recurrent proliferation of cork oak somatic embryos took place in a gelled medium without plant growth regulators (PGRs). Secondary somatic embryos mainly developed from meristems that appeared on the surface of a white compact proliferative tissue formed from the root cap of primary embryos, following a multicellular budding pathway (Puigderrajols et al. 1996). These primordial structures developed into cotyledonary embryos that were joined at their root poles forming embryo clusters.

Hence, the protocol for SE of adult cork oak trees gave rise to structures that differed from those generally considered useful to establish an embryogenic suspension. The inoculum to initiate an embryogenic suspension should be friable so that it easily disaggregates in the liquid medium (Strosse et al. 2006). In other studies, it has been demonstrated that selection of the proper starting material was essential for establishing suspensions (Burns and Wetzstein 1997; Tiwari et al. 2007).

In embryogenic cultures of cork oak, the formation of individual embryos of possible unicellular origin has also been described (Puigderrajols et al. 2001). This type of embryo appeared at a low frequency and its formation was associated with necrotic zones of proliferative tissue. Adjacent to these necrotic areas, the proliferative mass was more friable and of a brownish colour. From this mass, cells and small cell clumps could be isolated and when cultured in liquid medium developed embryos (Puigderrajols et al. 1996).

Plant regeneration through SE has been achieved in many *Quercus* species (Vieitez et al. 2011) but, to date, methods for initiating suspensions have not been described in detail (Jiménez et al. 2011). The present study was designed to establish embryogenic suspension cultures of cork oak using the embryo clusters generated during the recurrent stage of proliferation on gelled medium. Our objectives were:

1. To establish the starting explant suitable for initiation of embryogenic suspensions, by studying:
   (a) the effect of the culture duration of embryo clusters on the quantitative and qualitative characteristics of the materials present in the suspension.
   (b) the characteristics of the materials released when the embryo clusters are briefly shaken in liquid medium, and the capacity of those tissues that detach from embryo clusters to proliferate and generate embryogenic suspensions.
2. To develop a protocol for the maintenance of cork oak embryogenic suspensions obtained by culturing small clumps.
3. To determine the applicability of the developed methods to a range of genotypes, by studying the effects of genotype on the initial stages of the growth in suspension.

Materials and methods

Plant material and culture conditions

Cultures were established using embryo clusters from 12 embryogenic lines. These lines were obtained by inducing SE in expanding leaves of 100-year-old cork oak trees from different Spanish regions (Hernández et al. 2003a). Lines ALM2, ALM3, ALM5 and ALM80 were obtained from select trees on the estate La Almoraima (Cádiz); lines TRG1, TRG3, GUI3, GUI4, GUI5, ADB1 and ADB2 were from select trees in the Extremadura region; and line MEC15 from a single endangered tree on the Island of Minorca. The lines were maintained by recurrent embryogenesis on medium lacking plant growth regulators for 3–5 years. To address objectives 1 and 2, only genotype ALM80 was used. The remaining 11 lines were used for objective 3.

The culture medium was the same as used by Hernández et al. (2003b) in the proliferation stage. It contained the macronutrients of Schenk and Hildebrandt's (1972), the micronutrients, cofactors and Fe-EDTA of Murashige and Skoog's (1962) and 30 g l^{-1} sucrose, and was PGR-free. The pH was adjusted to 5.75 and the medium was autoclaved at 121°C for 30 min. In the experiments requiring gelled medium, 0.6 % agar was used (B&V S 1000 Parma, Italy).

Liquid cultures were established in 150-ml baffled Erlenmeyer flasks with 70 ml of medium. The flasks were

closed with a double layer of aluminium foil without any sealant. The cultures were agitated at 110 rpm on horizontal orbital shakers (30-mm radius of movement) at 25 ± 2 °C, 16-h photoperiod and 180 μmol m^{-2} s^{-1} light intensity. For the gelled medium cultures, baby food jars (Sigma®, 200 ml) with polypropylene lid and sealed with Parafilm® were used (Hernández et al. 2003b).

Initiation of embryogenic suspensions

Inoculation with embryo clusters: effect of culture duration on the morphology of suspensions

To characterize the effects of culture duration on the quantitative and qualitative characteristics of the suspensions, flasks were inoculated with 7 g of embryo clusters. Cell density and the number of aggregates measuring 41–800 μm were monitored over time. Cell density (number of cells ml^{-1}) was determined using a method similar to that described by Fujimura and Komamine (1979). In brief, samples of the suspension were withdrawn from different flasks at 2, 4, 6, 8 and 10 days after initiation by shaking the flasks and immediately pipetting out 5 ml using 800 μm-diameter pipette tips. Samples from four flasks (replicates) were taken at each time point. Cell counts were conducted in triplicate in 1-ml subsamples of each replicate, using a Bürker Brand chamber with a sample area of 9 mm^2, and a binocular microscope with magnification 40×. Both individual cells and those forming small clusters of up to five cells were counted. To determine the number of cells per ml the following equation was used:

$$\text{Cells/ml} = \frac{\text{cells counted} \times 1{,}000\,\text{mm}^3}{\text{area counted (mm}^2) \times \text{chamber depth (mm)} \times 1\,\text{ml dilution}}$$

To monitor changes in the number of structures measuring 41–800 μm, the flasks were sampled at 7, 14 and 21 days of culture. In this case, samples from six different flasks (replicates) were taken at each sampling time. At each time point, 5 ml of suspension was collected as described for the cell density determinations. Then each sample was filtered through a nylon Millipore® sieve (NY4104700) of pore size 41 μm. Any structures retained by the filters were counted by direct observation under a stereo microscope. In addition, 1-ml samples of the suspensions taken after 6 days of culture were stained with 1 % acetocarmine following the procedure described by Gupta and Durzan (1986) to determine the characteristics of the different cells and cell clumps.

Inoculation with cells and cell aggregates released from embryo clusters after brief shaking: characterization and proliferation capacity

Seven grams of embryo clusters grown on semisolid medium was deposited into ten flasks (7 g per flask) each containing 70 ml of liquid medium and shaken vigorously by hand. The cells and cell aggregates released into the medium were then used to initiate the suspensions. The larger size fragments were removed using a 800-μm steel sieve and the medium with the smaller size fraction was filtered in a sequential manner through nylon meshes of pore size 41 μm (Millipore® NY4104700) and 11 μm (Millipore® NY1104700). The fractions of 41–800 μm, 11–41 μm and less than 11 μm were separately used as the inocula. The flasks inoculated with fractions under 11 μm and 11–41 μm were cultured over 8 weeks, while those inoculated with the fraction 41–800 μm were cultured for 4 weeks. These cultures were sieved and separated into >800, 800–180 and 180–41 μm size ranges that were characterized and counted. There were ten replicates for each fraction.

To determine the embryogenic capacity of the aggregates, the aggregates of 180–800 and >800 μm were separated and cultured in gelled medium under conditions described for the proliferation stage (Hernández et al. 2003b). Embryo differentiation was assessed after 15 and 45 days.

Maintenance of the suspensions

When cultures were initiated using the fraction 41–800 μm that detached from embryo clusters, different types of EMCs were produced. To assess their proliferation capacity, two experiments were conducted. In the first experiment (1), single EMCs were used as inoculum (one EMC per flask) and the effects of EMCs size and morphology on proliferation were determined. A factorial design was used with three levels for the shape of the materials (spherical, nodular or oblong) and two levels for size (small 0.8–1.2 mm or large 2.5–4.5 mm). In addition, large-sized oblong EMCs were subjected to further tests using two inoculum types: EMCs showing secondary proliferations and EMCs with no evident secondary structures. For each inoculum type, six replicates were run. The proliferation capacity of each type and size of inoculum were evaluated by counting new growth over a 4-week culture period. The

new structures formed were fractionated into the size groups of >800, 180–800 and 41–180 μm, and the number of structures within each size range was determined.

In the second experiment (2), eight flasks were inoculated with four spherical, small (0.8–1.2 mm) EMCs each and the proliferation capacity was assessed as previously described. This inoculation procedure was finally adopted to maintain the liquid cultures and used for successive subcultures conducted every 4–6 weeks. To determine the possible effects of subculturing on proliferation capacity, this variable was also assessed after ten subcultures.

Initiation of suspensions with 11 genotypes of cork oak

To initiate the suspensions of 11 cork oak genotypes, the clumps of 41–800 μm in size that were released by shaking the embryo clusters were used. After 4 weeks of culture, the old medium was replenished with 70 ml of fresh medium and the cells were incubated for 4 weeks under constant shaking. At the end of the 8th week, the effect of the genotype was quantitatively assessed for: (1) proliferation potential, determined as the number of clumps larger than 180 μm; and qualitatively assessed for: (2) the biomass; (3) the ability to differentiate, determined by the presence of cotyledonary embryos and polar structures; and (4) the ability for maintaining suspensions, determined by the presence of embryogenic clumps of 0.8–1.2 mm opaque white or translucent with smooth surfaces. This assessment was independently undertaken by three observers who subjectively assigned to each culture a relative score for these characteristics. They used a five-point scale (very low, low, intermediate, high and very high). Inoculation with four spherical, small (0.8–1.2 mm) EMCs was used to test the possibility of maintaining the suspensions of the different genotypes.

Statistical analysis

The effects of culture duration on the cell density of the suspensions and on the number of embryogenic materials measuring less than 800 μm were assessed by analysis of variance (ANOVA) after testing normality by using the Kolmogorov–Smirnoff test. ANOVA was also used to compare the proliferation capacity of EMCs of different shape and size, to test the effects of the presence/absence of secondary embryogenesis in the inocula and the effect of successive subcultures on proliferation capacity. All statistical tests were performed using the GLM procedure implemented in the software package SPSS 13.0 for Windows. The significance level was set at $p \leq 0.05$.

Results

Embryogenic suspension initiation

Inoculation with embryo clusters: effect of culture duration on the suspension morphology and composition

When the cork oak embryo clusters were transferred from the gelled to the liquid medium, the embryonic tissues expanded and development of the cotyledons was observed (Fig. 1a). This process of enlargement of embryos and associated proliferative masses was accompanied by the appearance of necrotic tissues, release of cells and small cell clumps (Fig. 1b, h) and rapid darkening of the medium.

In the samples taken after 6 days of culture, individual cells and cell clumps were identified. These cells were isodiametric and small (20–30 μm) with a dense cytoplasm and sometimes a thick cell wall (Fig. 1c, d). Other cell types were also observed including larger, vacuolated forms with a globular or sinuous contour and large filiform cells. Organized structures were also present in the suspension, including 100- to 400-μm globular- and heart-stage embryos, with defined protodermis (Fig. 1e, f, g). Small structures (EMCs) detached from the embryo clusters comprised dense, translucent proliferative tissue and were of spherical shape with a smooth surface (Fig. 1h).

The suspensions significantly increased in cell density with the culture duration ($p < 0.001$). Two days after initiation, cell density was 0.98×10^6 cells ml^{-1} and remained the same until day 4, after which the number increased up to 4.3×10^6 cells ml^{-1} on day 6. Thereafter, cell density decreased and, after 10 days of culture, there were 2.2×10^6 cells ml^{-1} (Fig. 2a). From day 7 to 21 of culture, EMCs in the suspension showed linear fall in numbers. On day 7, an average of 4,170 EMCs per litre of culture medium were present, but by day 21 only 530 EMCs remained per litre of culture medium (Fig. 2b) due to the rapid degradation of EMCs that separated from embryo clusters.

Inoculation with cells and cell aggregates released from embryo clusters after brief shaking: characterization and proliferation capacity

When embryo clusters suspended in liquid medium were shaken, differently shaped and sized tissue pieces detached from the clusters. The smallest fraction of 11 μm was composed of cell fragments and a low number of very small isodiametric cells. The fraction 11–41 μm also contained cell fragments, cells and a few small groups of unorganized cell aggregates. When these two fractions

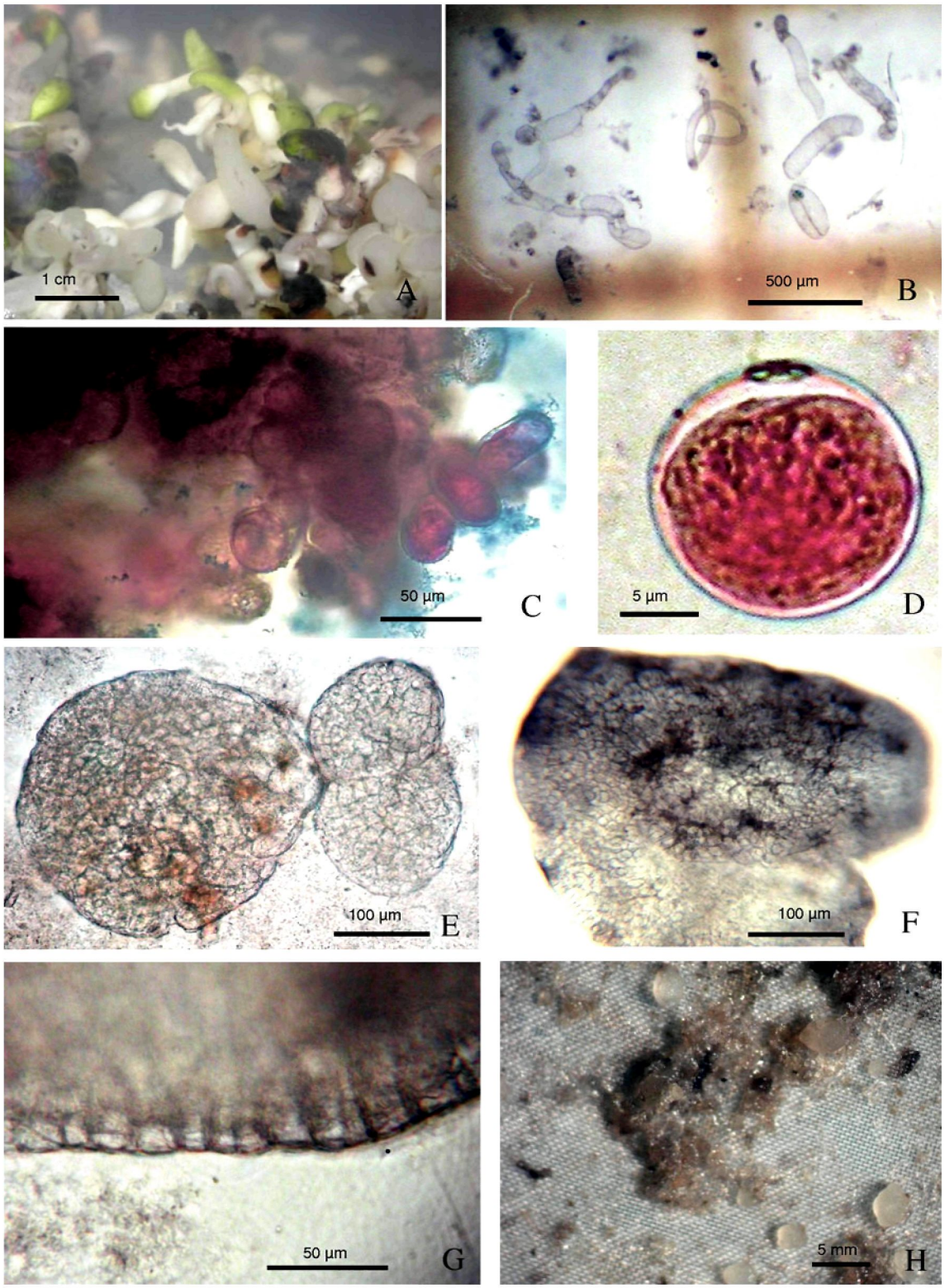

Fig. 1 **a** Embryo clusters after 14 days of culture in liquid medium. Note the developed cotyledons and the presence of necrotic tissues. **b–h** Suspension morphology 6 days after initiation. **b** General appearance of the suspension showing cell clumps, single cells and vacuolated cells. **c** Clumps of densely cytoplasmic isodiametric cells of 20–30 µm. **d** Isodiametric cell with a thick wall and high chromatin content. **e** Globular embryos. **f** Heart-stage embryo. **g** Details of the protodermis. **h** Spherical structures (EMCs) **c**, **d** are stained with acetocarmine

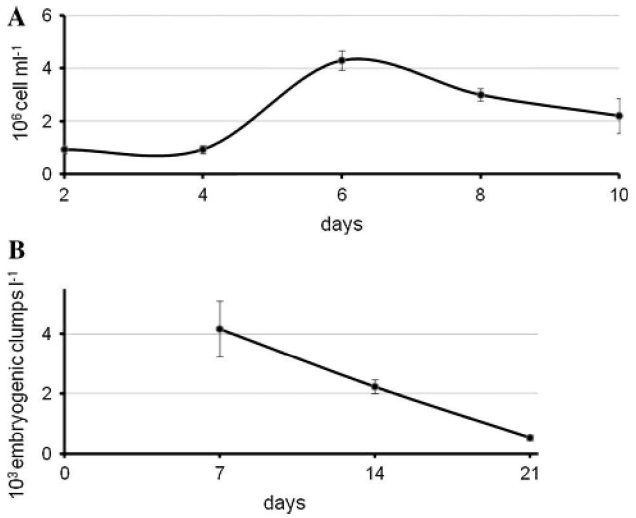

Fig. 2 Inoculation with embryo clusters taken from semisolid medium. **a** Changes in cell density during a 10-day culture period. Each point represents the mean of four observations. **b** Changes in the number of EMCs (41–800 μm) during a 21-day culture period. Each point represents the mean of six observations. *Bars* are standard errors

were cultured, no growth or proliferation was observed after 4 or 8 weeks of culture.

The fraction comprising cell aggregates of 41–800 μm that broke away from the embryo clusters was made up of cell remains, small groups of cell aggregates, EMCs with a smooth or slightly rough surface and embryos at early development stages. These populations were similar to those observed after 6 days in the suspensions produced by the prolonged liquid culture of embryo clusters described above (Fig. 1). Using this type of inoculum, a mean of 16,300 structures per litre of culture medium were generated after 4 weeks of culture. These structures were predominantly 41–180 μm in size and the number of larger sizes (180–800 μm and >800 μm) was smaller. High variability among replicates was recorded (Table 1). Cells, small groups of cells with no defined structure, different sized and shaped EMCs and cotyledonary embryos were identified in the suspensions. EMCs comprised opaque white or translucent dense material with a smooth surface. The smaller ones were basically spherical and the larger ones showed simple spherical or oblong shapes and complex nodular forms.

When the EMCs from liquid culture were transferred to a gelled medium, their embryogenic capacity was expressed. Tissue clumps from both fractions 180–800 μm and >800 μm developed into somatic embryos (Fig. 3a). Fraction 180–800 μm initially formed seemingly individual embryos and small EMCs (Fig. 3b). Subsequently, both the embryos and EMCs gave rise to secondary embryogenesis and formed embryo clusters. The structures in the fraction >800 μm also developed into EMCs and embryo clusters (Fig. 3c).

Table 1 Initiation of suspensions

	Number of structures $l^{-1}(\times 10^3)$	CV (%)
Overall	16.3 ± 3.2	63
Size range		
41–180 μm	10.0 ± 2.9	91
180–800 μm	3.3 ± 0.7	65
>800 μm	2.9 ± 0.7	78

Number of structures per litre of medium within different size ranges (mean ± SE and coefficients of variation, CV) produced after 4 weeks of culture using as inoculum the fraction 41–800 μm obtained by brief shaking of embryo clusters

Numbers are means and coefficients of variation obtained for the set of ten observations

Embryogenic suspension maintenance

Suspensions initiated with the fraction 41–800 μm released from embryo clusters contained heterogeneous tissue populations including EMCs. When single EMCs were cultured, great variability in the number of structures and their size distributions was observed. ANOVA revealed that these differences were not significantly correlated with EMCs size (large vs. small), shape (spherical, nodular or oblong) or with the presence or absence of recurrent embryogenesis in the initial inoculum. For the series of treatments, a mean of 22,200 structures were produced per litre of culture medium (Table 2) with a size range distribution and coefficients of variation similar to that obtained when the inoculum was the mixture of tissue pieces detached from the embryo clusters. When cultures were initiated with four EMCs instead of a single EMC, the number of structures produced within each of the three fractions tested was very similar, but the variation among replicates decreased considerably (Table 2). The inoculation of four EMCs per vessel (equivalent inoculum density 43 mg l^{-1}) generated a mean of 25,100 structures per litre of medium after 4 weeks of culture with a 31 % coefficient of variation. After ten subcultures using four EMCs as initial inoculum for each subculture, no variation was observed in the number of structures produced within each fraction, or in the variability among replicates (Table 2).

Although inter-replicate variability was reduced, the heterogeneity within each replicate remained independent of whether flasks were inoculated with one or four EMCs, and persisted after successive subcultures. The mean distribution in size groups of the materials produced per vessel was similar to that obtained in the initiation phase using the fraction 41–800 μm as initial inoculum. Around 60 % of the structures obtained per vessel were of size 41–180 μm, 22 % were 180–800 μm and 18 % were larger than 800 μm (Table 2). Inoculations of liquid medium with four

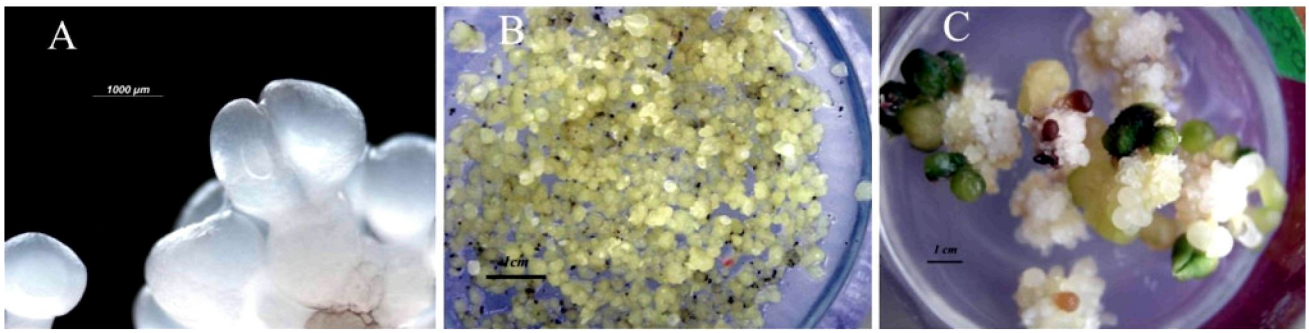

Fig. 3 Proliferation of structures in gelled medium transferred from liquid medium. **a** Details of a cotyledonary embryo at an early development stage. **b** Proliferating aggregates obtained after 15 days of culture using the fraction 180–800 µm; note single somatic embryos and small embryogenic masses. **c** Embryogenic masses and embryo clusters obtained after 45 days of culture using a fraction larger than 800 µm

Table 2 Maintenance of suspensions

	Inoculum characteristics					
	One EMC[a]		Four EMCs[b]			
			Initial culture		Ten subcultures	
	Number ($\times 10^3$)	CV %	Number ($\times 10^3$)	CV %	Number ($\times 10^3$)	CV %
Overall	22.2 ± 3.7	108	25.1 ± 2.8	31	24.1 ± 2.9	35
Size range						
41–180 µm	12.9 ± 1.8	96	15.3 ± 1.5	28	14.0 ± 1.8	36
180–800 µm	4.0 ± 0.6	93	5.7 ± 0.6	27	6.2 ± 0.6	28
>800 µm	5.1 ± 0.8	106	4.1 ± 0.5	33	3.7 ± 0.4	28

Number of structures per litre of medium within different size ranges (mean ± SE and coefficients of variation, CV) obtained after 4 weeks of culture using one or four embryogenic clumps (EMCs) as inoculum and after ten subcultures using four EMCs

[a] No differences with respect to size, shape or presence/absence of prior proliferation in the embryogenic clumps. Numbers are means and coefficients of variation obtained from 42 observations

[b] Means and coefficients of variation of eight observations

small EMCs resulted in the conservation of embryogenic capacity over ten subcultures.

Initiation of suspensions with 11 genotypes of cork oak

The biomass indicators or the number of clumps per vessel after 8 weeks of culture was variable across the 11 genotypes. Variability was evident both among genotypes and among replicates in each genotype. Depending on the genotype, a mean of 161–4,140 structures larger than 180 µm were produced per litre of culture medium (Table 3). On the other hand, the morphology, differentiation level and dispersal of small structures were highly linked to the genotype. The genotypes that had the greatest proliferation capacity by releasing a high number of EMCs into the medium were ALM80, ABD2 and TRG1. Genotype GUI5 showed a greater tendency to develop cotyledonary embryos and differentiated structures, and ALM2 generated the lowest amount of biomass (Table 3). As an example, Fig. 4 provides images of the initial proliferations obtained in liquid medium for genotypes ALM2, GUI5 and ABD2. For all genotypes, at least in one of the replicates, the presence of small dense aggregates that were potentially useful for maintaining the suspensions was observed (Table 3). Using these aggregate suspensions, all genotypes were maintained for two cycles of 4 weeks.

Discussion

When embryo clusters were used as initial inoculum, cotyledons and proliferation masses attached to embryos enlarged. An analogous response was described when mature embryos of onion were cultured in liquid medium

and the formed callus was not friable enough for initiating suspensions (Tiwari et al. 2007). With time, tissues became brown and symptoms of necrosis were evident. The changes observed in the cork oak suspensions were similar to those described by Burns and Wetzstein (1997) also for liquid cultures of embryo clusters of pecan (*Carya illinoinensis*). In the samples taken after 6 days of culture, individual cells and cell clumps were identified that showed features typical of potentially embryogenic cells (Fujimura and Komamine 1979, 1980; Georget et al. 2000; San José et al. 2010). In addition, other cell types were also observed including larger, vacuolated forms with a globular or sinuous contour and large filiform cells. These latter cells have often been observed in this type of suspension, but usually lacked embryogenic potential (Fujimura and Komamine 1979; Nomura and Komamine 1985; Finer 1994)

Table 3 Characteristics of embryogenic suspensions of 11 genotypes of cork oak

Genotype	Growth			
	Proliferation	Biomass	Differentiation	EMCs for maintaining
ALM2	579 ± 304	− −	−	+
ALM3	131 ± 74	++	++	−
ALM5	751 ± 296	+	+	+
ALM80	3346 ± 715	++	+++	+++
MEC15	313 ± 116	++	++	−
ABD1	1176 ± 747	+	+	+
ABD2	1931 ± 839	++	+	+++
GUI4	686 ± 252	+	+	+
GUI3	672 ± 593	+	++	++
GUI5	495 ± 217	++	+++	+
TRG1	4140 ± 701	++	−	+++
TRG3	1602 ± 733	+	−	++

Number of structures larger than 180 µm produced per litre of medium (proliferation, mean ± SE) after 8 weeks of culture using the fraction 41–800 µm as inoculum. The inoculum was obtained by brief shaking of embryo clusters

− − very low, − low, + intermediate, ++ high, +++ very high

The changes in cell density in the cork oak suspensions were similar to those described for cultures initiated from small clumps of carrot cells (Osuga and Komamine 1994). Although the carrot suspensions differed markedly in terms of both the nature of the starting plant material and differentiation processes, a 3- to 4-day lag phase followed by multiplication that persisted until 7th day was also noted. In the present work, once cell density reached a peak, cell numbers fell over time because of cell lysis.

The high level of differentiation and development attained by the embryonic tissues when embryo clusters were cultured in liquid medium might have been detrimental to the growth of these suspensions. Ideally, it would be desirable to achieve a culture in which inoculum growth is produced through cell proliferation without differentiation (Finer 1994). The cork oak embryo clusters showed considerable growth that was linked to differentiation processes, but this did not prevent the generation of suspensions containing a high diversity of potentially embryogenic populations smaller than 800 µm. Among these, individual cells or cell aggregates with no organization, dense proliferative structures of different shapes and embryos at early development stages were observed. However, as the culture duration increased, signs of necrosis and darkening of the medium were evident. These phenomena are generally associated with the buildup of

Fig. 4 Growth characteristics of the initial proliferations obtained in liquid culture for the genotypes ALM2, GUI5 and ABD2. The ALM2 genotype produced low biomass, low differentiation and intermediate proliferation values; GUI5 produced an intermediate biomass and high differentiation and proliferation rates; and ABD2 produced an intermediate biomass, low differentiation and very high proliferation

phenolic compounds and their oxidation that usually negatively affect the viability of cultures, especially when working with tissue explants of mature woody species (Benson 2000). On the other hand, the presence of phenols and signs of necrosis have also been linked to the formation of somatic embryos in woody species such as *Juglans* (Tulecke and McGranahan 1985), *Coffea* (Neuenschwander and Baumann 1992) or *Feijoa* (Reis et al. 2008). Another example is *Quercus suber*, for which the formation of singularized embryos in gelled medium has been associated with the necrosis of proliferative tissues (Puigderrajols et al. 1996). In liquid cultures of cork oak embryo clusters, as necrosis and phenolization of the medium progressed, the suspensions contained a diminished number of potentially embryogenic structures. Thus, as the culture duration increased, a decrease in both the number of cells or cell aggregates and the number of more organized structures comprising proliferative tissue was observed. Moreover, through cell lysis and degradation of proliferative structures, mucilaginous substance started to build up similar to that reported in pecan (Burns and Wetzstein 1997) making the suspensions difficult to fractionate due to clogged sieve pores.

When single cells and cell aggregates that comprised the smaller fractions released from the embryo clusters (after brief shaking) were used as initial inoculum, they barely grew. The limited proliferation capacity of these fractions might be linked to the nature of their components (partly cell debris) and also to the separation of individual cells and small cell aggregates (Vasil 2008). The fact that the minimum inoculum density was not reached (Strosse et al. 2003) and the absence of PGRs in the culture medium (Nomura and Komamine 1985; Osuga and Komamine 1994; Wong et al. 2006) could also be the cause of the lack of growth. In contrast, when the fraction 41–800 μm was used as inoculum, a high number of embryogenic structures were produced. Thus, cork oak suspensions belong to a few cases where proliferation in liquid cultures lacking PGRs has been reported (Burns and Wetzstein 1997).

Suspensions initiated with the largest fraction released from embryo clusters produced EMCs. These EMCs resembled the populations described by Finer (1994) as appropriate for the maintenance of suspensions according to the "low inoculum rule", referring to the low-density inoculation of dense cell clumps. Proliferations were heterogeneous even when EMCs of similar morphology and small size were individually cultured. It is likely that despite their similar external appearance, embryogenic clumps of 0.8–1.2 mm differed in their cellular composition and physiological properties. Accordingly, the use of an inoculum comprising several small EMCs reduced variation among replicates. The number of structures within different fractions remained stable regardless of whether flasks were inoculated with one or four EMCs, and persisted after successive subcultures. A stable ratio among different tissue types was also observed in pecan (Burns and Wetzstein 1997). The stable presence of material of different size may be the result of asynchronous processes of differentiation and development produced at the same time in the cultures.

When embryogenic tissues were transferred from liquid onto semisolid medium, they proliferated as recurrent cultures. These cultures behaved similarly as in the standard semisolid medium (Hernández et al. 2003b) and were able to produce germinable embryos.

Conclusions

There are a few studies reporting on embryogenic cultures that can proliferate for years on media without plant growth regulators. Cork oak is one of the species in which embryogenic lines of any genotype can be maintained in such a way. Although secondary embryogenesis takes place when embryos reach an advanced stage of development and PEMs cannot be obtained in semisolid medium, suspension cultures have been obtained from all the tested genotypes. There are also a few studies dealing with the initiation of suspensions using differentiated tissue clumps. In cork oak, the best initial suspension inoculum was obtained after brief shaking of the embryo clusters in liquid medium that were taken from semisolid cultures. This highlights the great embryogenic potential of the tissues that detach from the proliferating somatic embryos. Besides, suspensions could be maintained by transferring four small EMCs into fresh medium from which hundreds of embryogenic structures were produced in 4 weeks. These SE protocols open the door for the mass production of cork oak somatic seedlings to implement multivarietal forestry in this economically important species.

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